Development of Sensitive and Cost-effective ELISA based System for Detection of Aflatoxin Biomarker in Blood of Humans and Livestock

Bommakanti Ananth Srinivas
M.Sc. (Environmental Science & Technology)

CENTRE FOR ENVIRONMENT
Institute of Post Graduate Studies & Research
Jawaharlal Nehru Technological University
Hyderabad-28

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"Development of Sensitive and Cost-effective ELISA based System for Detection of Aflatoxin Biomarker in Blood of Humans and Livestock"

By
Bommakanti Ananth Srinivas

Submitted in partial fulfillment of the requirements for the Degree of

Master of Science
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International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. 502 324

Jawaharlal Nehru Technological University (JNTU), Masab tank, Hyderabad, India. 500 028

CGIAR

International Crops Research Institute for the Semi-Arid Tropics

ıeru 502 324
hra Pradesh
India

Tel +91 40 3296161 (19 lines)
Fax +91 40 3241239
+91 40 3296182
Email ICRISAT@CGIAR.ORG

CERTIFICATE

Mr. BOMMAKANTI ANANTH SRINIVAS has satisfactorily prosecuted the course of research and the thesis entitled " **Development of sensitive and cost-effective ELISA based system for detection of Aflatoxin Biomarker in blood of Humans and Livestock**" submitted is the result of original work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

(Dr. FARID WALIYAR)
Project Coordinator.

CERTIFICATE

This is to certify that the thesis entitled **"Development of sensitive and cost-effective ELISA based system for detection of Aflatoxin Biomarker in blood of Humans and Livestock "** submitted in partial fulfillment of the requirements for the degree, **Master of Science in Environmental Science and Technology** of Center for Environment, **Jawaharlal Nehru Technological University,** Hyderabad, is a record of the bonafide research work carried out by **Mr. BOMMAKANTI ANANTH SRINIVAS** under my supervision.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. The author of the thesis has duly acknowledged all the assistance and help received during the course of the investigation.

(Dr. FARID WALIYAR)
Project Coordinator.

Approved by:

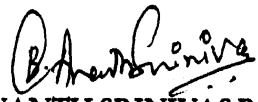
Prof. Y. ANJANEYULU
Head, Centre for Environment,
I.P.G.S.R,
Jawaharlal Nehru Technological University,
Masabtank, Hyderabad.

Centre for Environment
J.N.T.U. Hyderabad-500 028

External Examiner
(Prof. V. VENKATESWARLU)

DECLARATION

I hereby declare that I have independently carried out the dissertation work embodied in this thesis entitled, **"Development of Sensitive and cost effective ELISA based system for Detection of Aflatoxin Biomarker in Blood of Humans and Livestock"** under the supervision of **Dr. Farid Waliyar,** Principal Scientist, International Crops Research Institute for Semi Arid Tropics, Patancheru, Hyderabad. This work is original and has not been submitted for the award of any other degree or diploma on similar title for any other University or Institution.



ANANTH SRINIVAS BOMMAKANTI

PREFACE

ENVIRONMENT, DIET, AND CANCER – THE THREE MUSKETEERS: Development of simple and cost effective ELISA for detection of aflatoxin biomarker in blood of humans and live stock.

Food safety is one of the most important aspects of Environmental Health. In developing countries, hardly 60% of the population have accesses to safe and uncontaminated food. Aflatoxins are one of the common food contaminants that are found in almost all the food commodities right from maize, groundnut, chillies, rice, cotton, pepper, coriander, and in milk (metabolite of AFB1). Experimental evidence shows that aflatoxins are potent carcinogens, mutagens and teratogens. It is also proved that these aflatoxin act synergistically with hepatitis b virus in the etiology of liver cancer.

Due to lack of awareness among people about the ill effects of aflatoxins on health and correlation between authorities to monitor the level of aflatoxins in food makes them an unavoidable part of diet, particularly in developing countries. To protect the human health, estimation of aflatoxin exposure is very important so that strategies can be devised to mitigate the extent of exposure. Assessing dietary exposure to aflatoxin is particularly difficult by measuring intake of contaminated food, due to huge errors in sampling and given the fact that aflatoxins are present in almost major cereals and other dietary items which form a regular human diet. Therefore, exposure to aflatoxins can be done by estimating the amount of aflatoxin albumin adducts in blood or by measuring the aflatoxin DNA adducts in urine. Among many methods available for estimation of aflatoxin adducts, immuno-chemical methods are simple cost effective and adaptable. The major requirements for application of immuno-chemical methods are high quality antibodies and methodologies to use these antibodies for estimation of aflatoxin albumin adducts. There fore this thesis is focused on development of immuno-chemical based Enzyme Linked ImmunoSorbant Assay(ELISA) technique for assessing aflatoxin exposure in humans and live stock.

This thesis is divided in to Five Chapters containing subsections. Chapters One and Two contain "**Introduction**" and "**Review of Literature**", Chapter Three contains the "**Materials and Methods** " that are followed to attain the objective. This chapter is further sub divided in to sections which elucidate the process of synthesis of Aflatoxin Albumin Conjugates, Aflatoxin lysine adducts, Production of Polyconal antibodies for aflatoxin-epoxide and development of "Enzyme Linked ImmunoSorbant Assay" for analysis of blood samples. **Results** of the experiments are discussed immediately after each experiment .While Chapter Four focuses on **Future prospects** of the work carried out and Chapter five enlists all the "**References**" done to bring up this thesis .

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I deem it a privilege to extol my profound etiquette and sincere feelings of gratitude to an illustrious, distinguished scientist and my Project Coordinator, **Dr Farid Waliyar**, Principal Scientist , Pathology , International Crops Research Institute for the Semi-Arid Tropics, Patancheru for his initiative, benevolence, constant encouragement and ready help which enabled me to overcome several stumbling hurdles during the period of investigation and also in the preparation of this thesis. Personally his grace and vibrant scientific temper has left an everlasting impression on me. I could not have accomplished the task of research and presenting the findings, but for the benign help and transcendent suggestions offered by him at every subtle level.

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Last but no way the least, I am in debt to my parents **Smt. Bala.B** and **Shri.Venkateswarlu.B** and my brothers **Adtiya** and **Abhinav**, especially my mother for all her words of encouragement when I needed them the most. They has made me laugh when I was feeling down and pushed me when I really deserved to be shoved during the tough times in my educational pursuit.

Ananth Srinivas Bommakanti

List of Abbreviations

µL	Micro liters
Ab	Antibody
Ab-Ag	Antibody-Antigen Complex
AF-	Aflatoxin
AFB!-DNA	Aflatoxin B1- DNA adduct
AFB1	Aflatoxin B1
AFB1- OVA	Aflatoxin B1- Ovalbumin
AFB1-BSA	Aflatoxin B1- Bovine Serum Albumin
AFB2-	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1-	Aflatoxin M!
AFM2	Aflatoxin M2
AFT-Lys	Aflatoxin – Lusine Adduct
Ag	Antigen
AP/ALP	Alkaline phosphatase
BSA -	Bovine Serum Albumin
CSF	Cerebro Spinal Fluid
DNA	Deoxyribose Nucleic Acid
ELISA -	Enzyme Linked Immunosorbant Assay
G	Guanine

GAR-IgG	**Goat Anti Rabbit –Immunoglobulin G**
HBV	**hepatitis B virus**
HPLC	**High Performance Liquid Chromatography**
HPRO	**Horse Radish Peroxidase**
IC_{50}	**Inhibitory concentration 50**
Ig A	**Immunoglobulin A**
Ig G	**Immunoglobulin G**
Ig M	**Immunoglobulin M**
LD_{50}	**Lethal Dose 50**
MCPBA	**m-chloro per Benzoic Acid**
ng	**nanograms**
OD	**optical density**
OVA	**Chicken Egg Albumin**
pg.	**picp grams**
RIA	**Radio Immuno Assay**
RNA	**Ribose Nucleic Acid**
rpm	**revolutions per minute**
T	**Thymine**
TD_{50}	**Toxic Dose 50**
TLC	**Thin Layer Chromatography**
VFA	**Volatile Fatty Acids**

CONTENTS

List of Figures

List of Tables

Chapter I

INTRODUCTION

1.1 BACKGROUND

Natural environment provides a number of hazards to humans such as radiation, pathogenic bacteria, viruses, fungi, and certain gases. It is often thought that natural substances are harmless. This is a myth. In fact, many natural chemicals or derivatives of those chemicals can be just as toxic as anthropogenic chemicals such as pesticides, drugs and other industrial solvents, to both humans and other live stock (Ames et al 1990). The major naturally occurring hazardous chemicals that have resulted in adverse health affects of human population apart from plants and other animals are natural food contaminants such as Mycotoxins and bacterial toxins.

Food safety is one of the main criteria of Environmental Health problems apart from sanitary quality of drinking water, solid waste management, etc. In developing countries, only a small percentage of the population has access to safe and reliable supply of drinking water and uncontaminated food. Food contamination occurs not only because of miss handling but also can be due to naturally occurring microorganisms such as bacteria and fungi. As the environment gets more polluted the risk of food contamination increases. The main fungi which pose problem are those which produce toxic secondary metabolites called Mycotoxins which are "**Natural Environmental Pollutants**" . Fungal spores are wide spread in the environment and favorable conditions like high humidity, and tropical temperatures add to their survival capacity. In developing tropical countries like Indian subcontinent, Africa, China, Philippines, Southeast United States of America etc, fungal contamination is very common, as the environmental conditions favor fungal growth (Musil et al 2001). Apart from the poor storage facilities of food crops, unscientific pre and post harvest practices add to the severity of the problem. The major mycotoxins that are presently causing threat to human health are Aflatoxins, Ochratoxin, Fumonisins, etc. Among these Aflatoxins are the

most important as they are proven mutagens, teratogens, and immune suppressants. Liver diseases associated with Hepatitis B viral infection including hepato-cellular carcinomas account for more than one million deaths annually and over one million new cases a year . Of the many risks involved in etiology of Hepatocellular Carcinoma exposure to carcinogenic aflatoxins is a main independent factor and is of particular importance in certain regions of Indian subcontinent, China and Sub-Saharan Africa , as favorable environmental conditions for fungal growth and aflatoxin production prevail all round the year (Beckingham et al 2001). Due to this risk aasessment of aflatoxin exposure is gaining due importance.

Measurement of aflatoxin exposure on the basis of food analysis is problematic, as a variety of dietary items can be source of these toxins, and also sampling errors in dietary aflatoxin exposure analysis, do not reveal the actual extent of aflatoxin exposure at population level. An alternative basis for estimating this exposure is to use individual biomarkers of exposure. These biomarkers provide a more objective and representative measure of aflatoxin exposure circumventing the above mentioned difficulties in developing reliable estimate from dietary assessment. This can be done through measurement of urinary aflatoxin metabolites and aflatoxin DNA adducts or by measuring the covalent binding of aflatoxin to serum albumin (WHO 1998).

Many methods help in measurement of aflatoxin biomarkers. The most commonly used are physicochemical assay methods such as High Performance Liquid Chromatography and Liquid Chromatography. These are laborious and require expensive instrumentation and cleanup of samples. Therefore, these methods are of limited use in routine exposure assessment. To overcome the difficulties with these physiochemical methods new immuno chemical methods have been developed. Serological methods that use specific antibodies are

gaining wide acceptance for quantitative estimation of aflatoxin exposure because of their sensitivity and specificity (Devi K T et al 1999).

The main aim of this study is to develop highly sensitive and cost effective ELISA based method for aflatoxin exposure assessment in both humans and live stock.

1.2 OBJECTIVES

Aflatoxin exposure to humans is practically unavoidable because of their wide occurrence in variety of dietary items. Although strict measures are taken by regulating the aflatoxin level in foods and feeds, they may enter the foods during storage also. So aflatoxin exposure assessment must be done regularly in high risk geographical areas, where environmental conditions favor their production.

Immuno chemical methods that use specific antibodies and suitable, sensitive assay systems which are cost effective are therefore vital to mitigate the risk of negative effects of aflatoxins on human and live stock health. The objectives of present study were there fore

- Synthesis of aflatoxin- albumin adducts
- Production of polyclonal antibodies against the aflatoxin –epoxide molecule
- Development of ELISA based test procedures for estimation of aflatoxin-albumin biomarkers in blood
- Aflatoxin exposure assessment of representative blood samples.

CHAPTER II

Review of Litrature

2.1 Mycotoxins

Naturally occurring toxicants produced by microorganisms, such as bacteria and fungi contaminate foods and feeds. These food born hazards pose a serious risk to human and other mammalian health. Among the most potent naturally occurring toxins mycotoxins produced by fungi play a lead role (Styern 1995). Mycotoxins are chemically diversified low molecular weight compounds produced by secondary metabolism of fungal genera such as Aspergillus, Penecillium, Fusarium, Altenaria and Claviceps over a variety of food stuffs. These mycotoxins exhibit a wide array of biological effects and individual mycotoxins can be mutagenic, teratogenic carcinogenic, embryotoxic, oestrogenic and immunosupressive.(Hohler et al 1998). Depending upon the quantities produced and consumed ,mycotoxins can cause acute to chronic toxicity. Health effects may include immunological effects, organ specific toxicity, cancer and in some cases death. Agricultural workers are also at risk dermal and respiratory exposure during crop harvest.

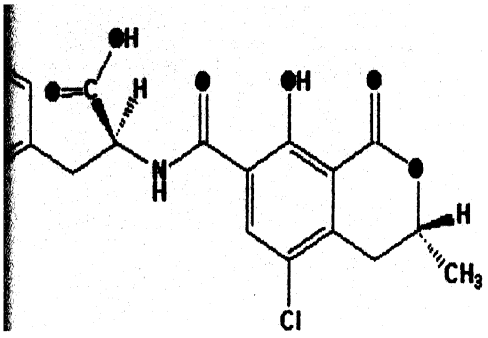
Several human mycotoxicoses have been described e.g., 'ergotism' whhich has been wide spread in europe and the far east from middle ages to 20th century; 'stachybotryo toxicosis' in the 1930's (Rodricks and Epply ,1974). And alimentary toxic leukemia in mid 1940's ,both in the soviet union.(Joffe 1971). The aflatoxin problem came in to light in 1960 when there was a severe out break of turkey x disease in England killing over 100,000 turkeys and other farm animals. The cause of this disease was traced to a feed component in peanut meal that was infested heavily with Aspergillus flavus. This single even ushered research on the mycotoxins. These studies included the occurrence, chemical structure , biosynthesis ,factors affecting the biosynthesis and health hazards of aflatoxins.

The mycotoxicoses are not only clinically diverse but also often extremely difficult to diagnose owing to the numerous pharmacological effects of causative mycotoxins.

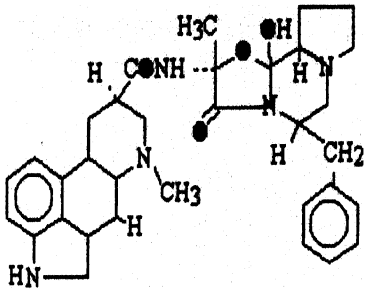
Helsh 1990 defined the criteria for human mycotoxicoses as follows.

- Occurrence of mycotoxins in food supplies
- Exposure to mycotoxins
- Co relation between occurrence and exposure
- Reproducibility of characteristic symptoms in experimental animals
- Similar mode of action in human and animal models.

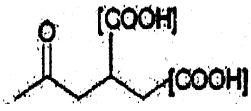
Until recently, dietary and occupational response were the primary of concern ,but with growing awareness towards the problem associated with indoor mould and respiratory exposures , researchers are recognizing the potential scope of mycotoxins exposures in broader than originally expected.



Ochratoxin .

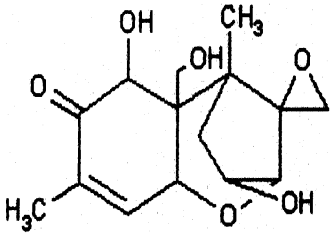


Ergotamine



R1 R2

CH3



[COOH]

Deoxynivalenol

Fumonisin

Table 1

The following table shows different Mycotoxins which occur naturally in foods and feeds.

Mycotoxins	Major producing Fungi	Substrate in nature	Biological effect
Alternarai mycotoxin	Alternaria alternata	Cerealgrains, Tomato,animal feeds	M Hm
Aflatoxins	Aspergillus flavus A.parasitius	Peanut,corn,cotton seed,cereals figs,milk,sorghum, walnuts	H, C, M ,T
Cyclopiazonic acid	A.flavus Penicillium cyclopium	Peanut,corn,cheese	Nr, Cv
Ochratoxin A	A.ochraceus, p.verrucosum	Barley,beans,cereals Coffee,feeds,maize, Rice,rye,wheat	Nh, T
Penicillic acid	P.puberulum A.ochraceus	Barley,corn	Nr, C, M
Zearalenone	Fusarium graminearum	Cereals.,corns,feeds rice	G, M
T-2 Toxins	F.sporotrichiodes	Corns,feeds,hays	D,ATA, T

ATA- Alimentary toxic aleukia ; C- carcinogenic ; Cv- cardiovascular lesion

D – dermatoxin ; G – genitotoxin and estrotoxic effect ; H – hepatotoxic

Hm – hemorrhagic ; M – mutagenic ; Nh – nephrotoxic; Nr – neurotoxic

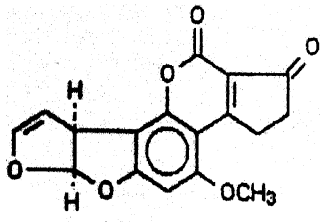
T- teratogenic

Source F S CHU, 1995

2.2 Aflatoxins and Chemistry

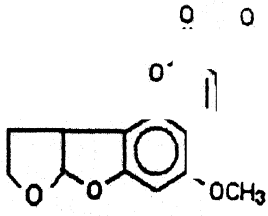
Aflatoxins in general are highly substituted coumarin containing fused hydro furo-furan moieties. Aflatoxins can be classified into two broad groups according to their chemical structure. The difurocoumarocyclopentanone series(AFB1, AFB2, AFM1,AFM2 and Aflatoxicol.)or B group toxins which were charecterised by fusion of a cyclo pentanone ring to lactone ring of the coumarin structure which fluoresce in blue colour under UV light and the difuranocumarolactone series (AFG1, AFG2 ,AFGM1 and AFB3) or G group toxins which contain an additional fused lactone ring. The G group toxins flourscе in greenish colour under UV light. Stucturally, the dihydro furan moiety, containing double bond, and the substituents linked to the coumarin are of importance in producing biological effects.(Steyn and 1995). Among all aflatoxin B1 is the major toxin and is produced by the fungi in large quantities. Pure aflatoxins are white crystaline solids.

Different structures of major aflatoxins along with chemical formulas can be seen in fig 2.



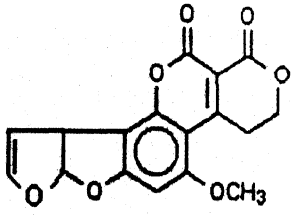
Aflatoxin B1

2,3,6a,9a-tetrahydro-4-methoxy-cyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]-benzopyran-1,11-dione



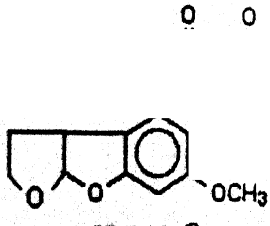
Aflatoxin B2

2,3,6a,8,9a-hexahydro-4-methoxy-cyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]-benzopyran-1,11-dione



Aflatoxin G1

3,4,7a,10a-tetrahydro-5-methoxy-1H,12H-furo[3',2':4,5]-furo[2,3-h]pyrano[3,4-c][1]-benzopyran1,12-dione



Aflatoxin G2

3,4,7a,9,10,10a-hexahydro-5-methoxy-1H,12H-furo(3',2':4,5]-furo[2,3-h]pyrano[3,4-c][1]-benzopyran1,12-dione.

2.2.1 OCCURRENCE.

Aspergillus species are ubiquitous in areas of world with hot and humid climates including Subsaharan Africa and Southeast Asia. Aflatoxins are produced on a variety of substrates at an optimum temperature of 30-35 ^{0}C and at a relative humidity of 80-85%. Among the substrates rice, groundnut, and maize have been shown to yield substantial amounts of aflatoxins under laboratory conditions(Hesseltine etal 1966). Aflatoxins are widespread since they are produced in both tropical and subtropical environments, pre and post harvest conditions and on a variety of crops due to ubiquitous nature of A.flavus making it difficult to control aflatoxin production.. The common dietary food substances which are susceptible to aflatoxin contamination are maize, rice, cotton , groundnuts, wheat, sorghum, chilli, pepper, almonds, walnuts , coriander, cheese etc.

2.2.2 ENVIRONMENTAL CONDITIONS AND AFLATOXIN PRODUCTION

Fungal growth and toxin production results from the interaction of numerous environmental factors. The initial contamination is mostly due to the presence of spores of Aspergillus in the soil. Some times air borne fungal spores, other grain or dockage also serves as the source of inoculum. Mechanical damage to the seed coat from harvesting equipment, insects and birds may also facilitate the infection. Available oxygen, time, temperature, and moisture content are main factors, which mediate the fungal growth.. Aflatoxin was found to be highest in the rainy season and lowest in the hot season (FAO 1979).High temepratures and relaive humidity favors the growth of fungus which in turn triggers the aflatoxin production. In Indian subcontinent aflatoxin is a major problem since droughts and floods occur alternatively in various states during the growing and harvesting seasons. Usually the temperatures are around 25 ^{0} C all-round the year, which provides a congenial environment for the toxin production. In addition the harvesting seasons usually fall on the onset of the

wet season. During this period, sun drying may not be possible which is the usual method employed by the farmers in India. Thus grain enters the storage system at high moisture content. Aflatoxin contamination is at its peak when drying is not done with in 48 hours (FAO 1979,1987). Further more , with the green house effect or global warming , alteration of the climatic conditions an most Asian countries has been observed and this is adding to the ailment (Musil et al 2001).

Production of aflatoxins is dependent on weather conditions. A dry growing environment or drought stress tends to favor the development of aflatoxin. When soil moisture is below normal and temperatures are high, the number of Aspergillus spores in the air increases. During pollination, these spores infect corn kernels either through silks (pollination tubes) or through areas of damage caused by insects, birds and weather events. Once infected, plant stress such as nutrient deficiency, continued dry weather or kernel damage during harvest may increase aflatoxin levels.

2.3Exposure to Aflatoxin

Diet is the major way through which humans as well as animals are exposed to aflatoxins. Apart from this, exposure to aflatoxin can be through ingestion of contaminated milk containing AFM1(metabolite of AFB1). Occupational exposure to aflatoxins in agricultural workers, people working in oil mills, and granaries have been reported Sorenson et al 1984)

2.4 Health Aspects

Epidemiological, clinical, and experimental studies reveal that exposure to large doses (>6000μg) of aflatoxin may cause acute toxicity with lethal effect whereas exposure to small

doses for prolonged periods is carcinogenic (Groopmann et al 1988) The adverse effects of aflatoxins on animal can be categorized into two general forms .

1. Acute Toxicity.
2. Chronic Toxicity.

2.4.1 Acute Toxicity

Acute toxicity is caused when large doses of aflatoxin are ingested. This is common in livestock. The principal target organ for aflatoxins is the liver. After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes and leads to necrosis or liver cell death. This is mainly because aflatoxin metabolites react negatively with different cell proteins, which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis. In correlation with the decrease in liver function, there is a derangement of the blood clotting mechanism, icterus (jaundice), and a decrease in essential serum proteins synthesized by the liver. Other general signs of Aflatoxicosis are edema of the lower extremities, abdominal pain, and vomiting. The most sever case of acute poisoning of aflatoxin was reported in north-west India in 1974 where 25% of the exposed population died after ingestion of the molded maize with aflatoxin levels ranging from 6250 to 15600μg/kg.

2.4.2 Chronic Toxicity

This is due to long term exposure of moderate to low aflatoxin concentration. The symptoms include decrease in growth rate, lowered milk or egg production, and immuno suppression. There is some observed carcinogenecity, mainly related to AFB1. Liver damage is apparent due to the yellow color that is characteristic of jaundice, and the gall bladder becomes swollen. Immuno-suppression is due to the reactivity of aflatoxins with T-cells, decrease in Vitamin K activities, and a decrease in phagocytic activity in macrophages. These

immuno suppressive effects of aflatoxins predispose the animals to many secondary infections due to other fungi, bacteria and viruses. (Robens et al 1992, Mclean 1995)

2.4.3Aflatoxin and Hepatitis.

Many experiments conducted in different areas especially in China and in the African countries, have shown high incidence of hepatitis B virus infection where dietary exposure to aflatoxins was prevalent. Subsequent research proved that both aflatoxins and hepatitis B virus act synergistically in the etiology of liver cancer (Montesano et al 1997, Groopman et al 1996.)

2.4.4 Aflatoxins and Animal Health

No animal species is resistant to the acute toxic effects of aflatoxins. A wide variation in LD_{50} values has been obtained in animal species tested with single doses of aflatoxins. For most species, the LD_{50} value ranges from 0.5 to 10-mg/kg body weight. Animal species respond differently in their susceptibility to the chronic and acute toxicity of aflatoxins. Environmental factors, exposure level, and duration of exposure beside age, health, and nutritional status of diet can influence the toxicity (FAO web library 2000).

Table 2

Acute toxicity of aflatoxin B1 expressed as a single oral dose LD50 (FAO web library).

Species	LD50 mg kg $^{-1}$ bodyweight
Rabbit	0.30
Duckling (11 day old)	0.43
Cat	0 55
Pig	0.60
Rainbow trout	0.80
Dog	0.50 - 1.00
Sheep	1.00 - 2.00
Guinea pig	140 - 2.00
Baboon	2.00
Chicken	6.30
Rat (male)	5.50 - 7.20
Rat (female)	17.90
Macaque (female)	7.80
Mouse	9.00
Hamster	10.20

2.4.5 Specific Species Affects (Reed et al, 1987).

Swine

Aflatoxicosis in swine is mainly due to the fact that corn is a large part of their diet. Studies show that 0.4 ppm in the diet, from weaning to market weight, will have negative effects on health and growth rate. This is due, in part, to the decrease in feed efficiency of the infected feed. Piglets are more susceptible than adults and it has been shown that feeding sows AFM 1, during lactation, can cause stunted growth in her litter.

Large doses of aflatoxins have been shown to produce hepatic necrosis. The effects of aflatoxicosis can be compounded with the addition of stress. This can lead to ataxia and induced hemorrhaging. The hemorrhaging is due to the prolonged blood clotting time caused by lack of Vitamin K utilization. Treatment with Vitamin K, menadione, and even protein supplementation have shown to have some proactive affects for reducing the effects of aflatoxicosis in swine.

Poultry

Aflatoxicosis has the same toxic effects in poultry as it does in mammals. A dose of 0.25 ppm in turkey pouts and ducklings impairs growth, and a dose of 1.5 ppm in broilers and 4 ppm in Japanese quail has a negative affect on growth. An increase in blood clotting time increases the susceptibility of the carcass to bruising even at doses below that to have an affect on growth. In poultry, aflatoxins impair the availability of bile salts, which decreases Vitamin D production. This causes a decrease in the absorption of fat-soluble vitamins. Aflatoxins also decrease the production of Vitamin A in the liver, and it has secondary effects such as decreased blood calcium levels, decreased bone strength, and a decreased tissue and serum tocopherol level. This decrease in tocopherol levels can lead to Vitamin A and E deficiencies.

Ruminants

The effects of aflatoxicosis in ruminants are similar to those of non-ruminants. Calves are more sensitive than yearlings and adults. A dose of 0.2 mg/kg body weight can cause a decrease in weight gains. This can be attributed to poor feed utilization and a dramatic increase in alkaline phosphate activity in the rumen. Chronic aflatoxicosis in adult ruminants can cause anorexia, drying and peeling of the skin on the muzzle, rectal prolapse, and abdominal edema. Aflatoxicosis has also been shown to cause decreased fertility, abortion, and lowered birth weights in sheep. Some evidence on aflatoxicosis shows an effect on rumen microflora. This is characterized by a decrease in cellulolysis, VFA production, and ammonium formation.

Rabbits

These are highly susceptible species to aflatoxins. The LD50 in rabbits was determined as 300μg/kg Aflatoxin contaminated feed cause hemolytic anemia in rabbits fed with a dosage of 15 mg/kg for 30 days. Strong cytotoxic effects are also seen (Verma et al, 1998).

2.4.6 Aflatoxin and Human health.

Aflatoxicoses in humans was reported in many countries like India, China, Thailand, and several African countries. In African and Asian countries, where environmental condition favor the aflatoxin contamination, threat to human health from aflatoxins is quiet high. Studies on aflatoxin exposure and incidence of liver cancer by Groopmann and Wild(1994-2001) in places like China and West Africa showed that the situation was alarming. (Aspen cancer conference, 2001).

Studies relating to aflatoxin exposure of humans in India are not being done actively. However, this problem is present and may break loose at any time in near future, as incidence of aflatoxin contamination in foods and feeds is very common.

Aflatoxin and children

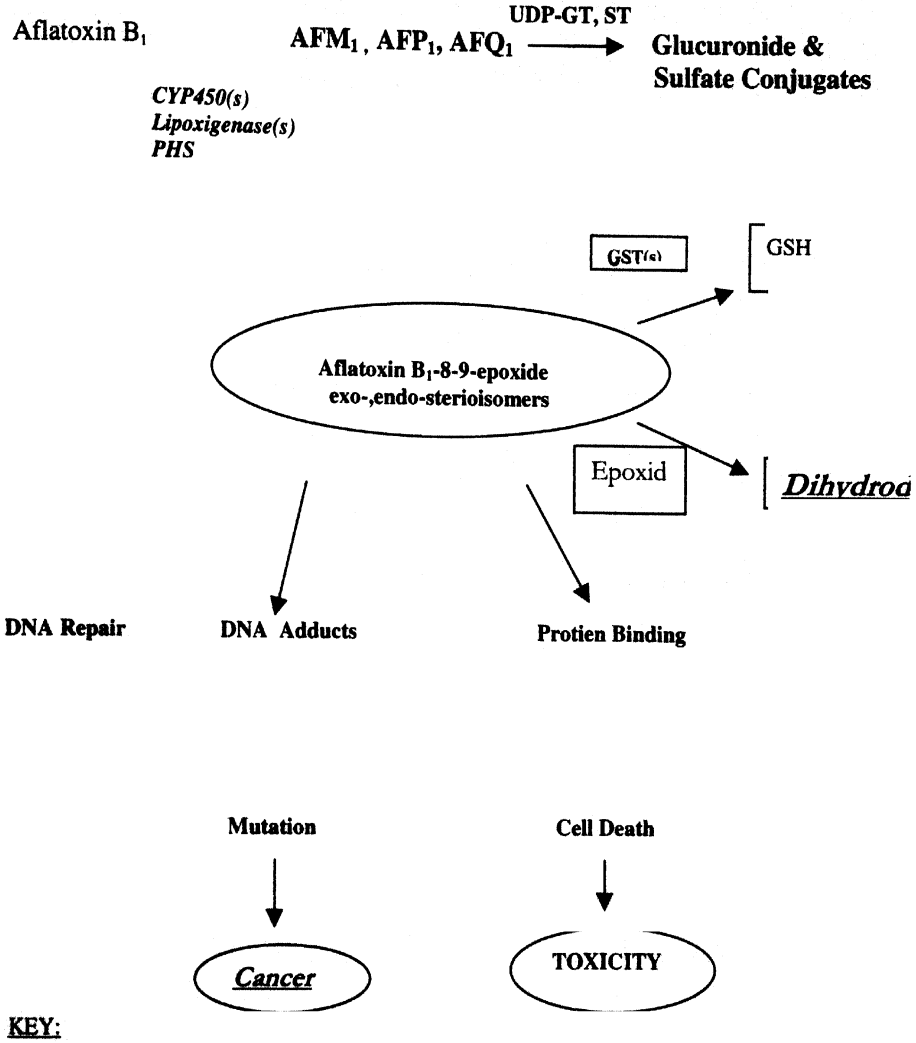
Foetal and childhood environment, including the nutritional status of the pregnant mother and the infant, are considered critical for growth and risk of disease in earlier life. Mal-nourishment is one of the common problems in developing countries. Apart from these, they are also exposed to high levels of mycotoxins. Aflatoxins are the major among these. It has been proved that these aflatoxins are immunogenic, teratogenic, and they retard the growth among experimental animals. In the developing countries like India and China, the environmental conditions favor their production. High exposure of these aflatoxins occurs through out these regions. A study in West Africa showed a significant correlation among the aflatoxin exposure and stunted growth in children who are exposed to aflatoxin right for neonatal stages (Gong et al 2002). Apart from that due to the capacity of aflatoxins to cross the placental barrier, can cause genetic defects at foetal stages itself (Maxwell et al 1998).

2.5 Metabolic Activation of Aflatoxin

After wide experimentation on many animal species like rats, rainbow trout's, aflatoxin especially aflatoxin B1 is confirmed as a potential carcinogen (IARC 1993). Metabolism plays a major role in deciding the degree of toxicity (Eaton et al 1994). After ingestion, aflatoxin is metabolized by cytochrome p450 group of enzymes in the liver, where it is converted to many metabolic products like aflatoxicol, AFQ1, AFP1, and AFM1, depending on the genetic predisposition of the species. Along with the above another metabolite called aflatoxin 8,9 epoxide is also formed. The amount of this metabolite decides the species susceptibility as this can induce mutations by intercalating in to DNA ,by forming an adduct

with guanine moiety in the DNA (Smela. et al 2001). This intercalation of Epoxide causes a G→ T transversion at codon 249 in p53 gene in liver, which may lead to hepatic carcinoma. This was observed in most of the experimental models, and it is presumed that this is the major reason for aflatoxin carcinogenecity (Katherine et al 1997 , Railey et al 97). Moreover species susceptibility to aflatoxin mainly depends on its liver detoxification systems, genetic make up, age and other nutritional factors (Howard et al 1990.). This Epoxide also forms adducts with serum proteins mainly albumin which serve as a potential biomarker for aflatoxin exposure assessment (Sabbioni et al 1987). According to Ames et al, 1990 only dioxins (TD_{50} = 6.7 X 10^{-6} mg/kg/d) significantly exceeds AFB1 (TD_{50}=9.3 x 10^{-4} mg/kg/d) in its potency. Using TD_{50} parameter AFB1 is 1000 times more potent as a carcinogen when compared to benzo pyrene (Eaton. et al 1997). IARC has classified Aflatoxin as a group one carcinogen (IARC 1983).

OVERVIEW of AFLATOXIN B_1 BIOTRANSFORMATION



KEY:

CYP450(s) – cytochrome P450(s)
PHS - prostaglandin H-synthatase
GST(s) - glutathione S – transferases
UDP – GT(s) - UDP – glucuronosyltransferases
ST(s) - sulfotransferases

SOURCE : **9.26, Aflatoxin toxicology , DAVID L. EATON and JOHN T. HEINONEN ,**

Comprehensive toxicology, Speir , McQueen , Gandolf Permagon Publications 1997.

2.6 Monitoring of Aflatoxin Exposure

Monitoring of human exposure to aflatoxins can be accomplished indirectly by dietary survey of raw agricultural commodities and food products ready for consumption. In practicality it is quiet difficult to monitor the dietary intake of a individuals as they are laborious, difficult and moreover have a high statistical coefficient in analysis (Sujatha et al 2001). There were several studies which aimed at establishing the incidence of primary liver cancer and aflatoxin exposure by measuring the contamination in food samples in Keneya , Swaziland, Thailand and the People Republic of China, the results obtained varied greatly form place to place and were need to be extrapolated (Yeh et al 1989). Such kind of data do not provide information on the biologically effective dose of aflatoxin at individual level i.e., the amount of activated agent that has actually reacted with critical cellular targets such as DNA, RNA or proteins, which is influenced by intake ,ditribution, metabolic activation /inactivation and excretion . It becomes particularly more important when considering the interaction with other known risk factors for primary hepatocllular carcinoma such as alcohol consumpution and environmental factors like HBV exposure , tobacco and other carcinogens.(Wild etal 1990). Thus biologically effective dose for any individual must not only reflect food intake but also host associated and environmental variants mentioned above. Such measurements have recently become feasible in the field of molecular and biochemical epidemiology due to development of sensitive assay methodologies.

The alternative approach is to estimate the aflatoxin biomarkers which are produced inside the body of exposed individuals. These biomarkers can be metabolic products which are excreted out in urine or fecal samples, milk or saliva. They can also be nucleic acid adducts or

protien adducts which are ususaly formed due to interaction between the toxin and respective macromolecules .

Among the desirable features for laboratoriy assay for AF exposure criteria like

- Indication of long term exposure
- Preferably non invasive method of sampling
- Replicability

must be satisfied (Wild et al 1990).

Several approaches have been used by many researchers to estimate aflatoxin exposure by measuring metabolites in urine and milk samples . The atudies were aimed at measuring different aflatoxin metabolites like AFM1, aflatoxicol, aflatoxicol M1 in urine and milk samples (Campbell et al1970 , Groopmann etal 1985) . But all these indicated recent exposure and does not particulay denote long term exposures. In recent past AFB1-DNA adduct and AFB1 albumin adduct have been used in both animal and human molecular dosimetric investigations. Both these adducts are formed due to metabolic activation of aflatoxin in the exposed individuals. Among these DNA adduct is excreted in urinary samples and can be obtained by non invasive methods. However may not serve as stable biomarker for long term exposures as this is usually repaired by the cellular DNA repair mechanisms. Covalent binding of aflatoxin to albumin was discovered 25 years ago by Dalezios et al 1972. Later Sabbioni etal 1987 isolated and charecterized the major aflatoxin albumin adduct from the blood of exposed animals. They analysed that aflatoxin majorly binds with lysine residues present on the surface of the albumin molecule.. They also they confirmed that halflife of this adduct was similar to that of albumin form normal individuals and this would serve as a stable biomarker for aflatoxin exposure assesment in humans.

Initial studies were aimed at directly analysing the albumin adduct in the blood. But the results obtained were not accurate and sensitive (Andrew et al 1987) Later Sabbioni etal 1990 measured Aflatoxin –Lysine adduct which was seprated form the serum albumin after hydrolysing it with proteinases. This mehtod proved to be very accurate and was far more sensitive with compared to the earlier studies.

Invirto epoxidation of Aflatoxin molecule was first done using liver microsomal enzymes. Then Baertschi et al 1988 synthesised Aflatoxin epoxide molecule using dimethyl dioxirane. Later Afb1-di bromo compound an analouge similar to Aflatoxin lysine adduct and was used for dosimetric investigations.(Wood et al 1988). Dimethyl dioxirane had a small shelf life and was toxic. Recently Sujatha 2001 synthesised Aflatoxin epoxide – lysine adduct similar to that of metabolic activation using m-chloro per benzoic acid and fully charecterized the molecule and validated its usage for ELISA. They achieved a molar ratio of 16:1 of Aflatoxin to lysine molecule. Procudure for synthesis of AFB1-BSA and AFB1-OVA were also proposed by the same group. The same procedure was adapted for carrying out this assay development.

2.7 Methods for AF-Exposure Assessment

Different methods were validated for assessing the aflatoxin exposure. Biological methods which are based on the toxic effect of mycotoxins have been used for detection utilizing either the death or pathological lesions or biochemical events or immuno-toxic effects as the index for toxicity. These methods were not specific and sensitivity is generally low when compared to other methods. Physico chemical methods such as thin layer chromatography, high performance liquid chromatography, gas chromatography, and mass spectrometric methods are laborious and require expensive instrumentation and clean up of samples. This limits their usage in routine laboratory scale.

To overcome the difficulties faced by biological and physicochemical methods new immunological methods were developed. Immunological methods using specific antibodies are gaining wide acceptance for aflatoxin exposure assessment because of their sensitivity and specificity and are less expensive to perform than the majority of analytical methods.

2.7.1 Thin Layer Chromatography (TLC)

TLC was the first method to be used for detection of mycotoxins in foods and feeds. Since this method was simple and does not need extensive instrumentation, it has remained important in many laboratories. This was one of the first methods used for analysis of aflatoxin metabolites in urine samples.. Campbell et al 1970 analysed AFB1 content by measuring different metabolites in urine sample. AFM1 was detected in measurable quantities. Later Lovelace et al 1982 published mehtods for separation of different metabolites of AFB1 using TLC. However, due to lack of sensitivity and high sample cleanup procedures, TLC was not considered as an option for measuring individual exposure for long term studies.

2.7.2 High Performance Liquid Chromatography (HPLC)

HPLC can be used to for detection of aflatoxin adducts to very low quantities. With advances in chemistry of adsorption materials for column packing, improvement in instrumentation, enhancement of efficiency of detectors as well as methods for both pre column and post column derivitization, both sensitivity and reproducibility have improved considerably over the years. Thus HPLC is considered as the most popular method for aflatoxin exposure assessment. But due to its laborious sample clean up mechanisms, expensive instrumentation, restricts its use in large-scale population studies (Wild et al 1990, Groopman et al 1999)

2.7.3 Immunological methods (**polyclonal** and **monoclonal** antibodies).

Polyclonal antibodies are cheaper to produce than monoclonal and also can be highly specific when made to highly purified antigen. Since Polyclonal antibodies consist of heterologous population of antibodies with variable specificities, they tend to be broadly specific and widely applicable to different serological tests. Therefore for routine detection polyclonal antibodies are highly suitable.

Polyclonal antibodies are obtained from serum of animal following injection with an antigen,which contains many antigenic sites. Therefore antibodies produced react with more than one epitope.

Choice of animals

Any warm blooded animal can be used for antibody production eg Rabbits, chickens,guinea pigs, rats , sheep, goats and horses. When small animals such as rats and mice are used, only small quantity of serum can be obtained. Although large animals such as goats and horses can provide large volumes of serum, large amounts of antigen are required for immunizing these animals. The rabbit is the most commonly used animal for antibody production.

Immunization

Injection of an antigen into an animal is accomplished either by intramuscular or subcutaneous or intravenous injections. For injection the antigen preparation should be emulsified with an adjuvant (1:1 proportion). The most commonly used adjuvant is Freund's adjuvant, which consists of paraffin oil and an emulsifier, mannide monooleate (incomplete). Complete adjuvant , in addition to these components, contain heat- killed *Mycobacterium tuberculosis* , or *M. butyricum* or a similar acid – fast bacterium. Emulsification with adjuvant

results in very slow release of antigen, there by stimulating excellent immune response. Antigen concentration required may vary from 100 µl /ml to 500 µl/ml. A normal immunization schedule followed for rabbits is given below

- Four intramuscular injections (multiple sites) at weekly intervals (for first injection Freund's complete adjuvant and further 2nd, 3rd and 4th use incomplete adjuvant). Five injections are usually adequate to obtain immune response.
- If the titer of the antibody is low, either an intravenous (for intravenous injection adjuvant should not be used) or an intramuscular injection should be given as a booster.

Blood collection and serum preparation

Blood is collected from rabbits by making an incision in the marginal vein of the ear. It is preferable to collect the blood in sterile containers about 15-20 ml for each bleed. The blood is allowed to clot at room temperature for 2-3 hrs. (This can also be done at exposure at 37°C for 30 min). After overnight refrigeration, the serum is collected with a Pasteur pipette and then centrifuged at 5000-rpm for10 min.

Note: It is important to starve rabbits for atleast 24 hrs before blood collection to minimize concentration of lipids.

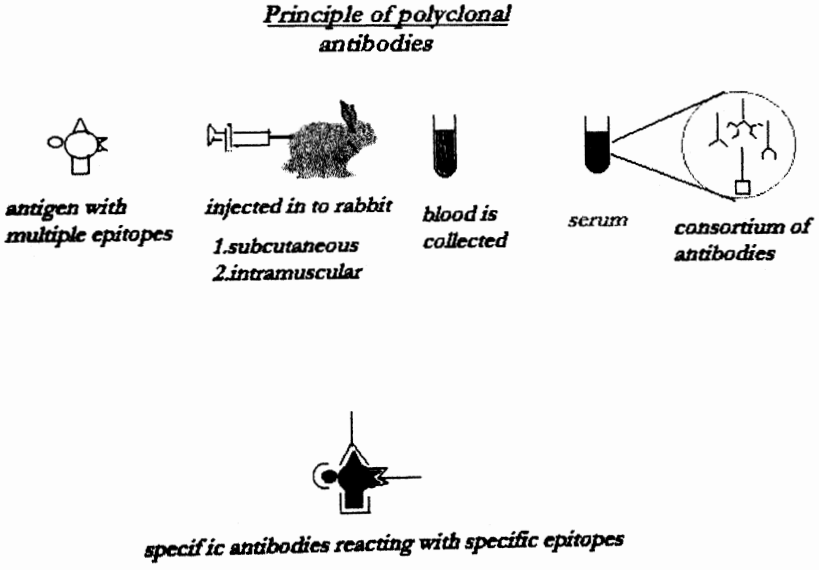
Storage of antiserum

- For long term storage of antiserum at 4oC, it is essential to add either glycerol (1:1) or sodium azide to a concentration of 0.02%.
- In lyophilized form, antisera can be stored at –20°c indefinitely. Antisera can be stored at – 70°c for many years without losing potency. Antiserum mixed with an equal volume of glycerol can also be stored at – 20°C.
- It is advisable to store serum in small aliquots of 1.0 ml or less.

- Antiserum should not be frozen and thawed repeatedly. This leads to aggregation of antibodies there by affecting antibody activity by steric interference of the antigen-combining site or by generating insoluble material, which may sediment during centrifugation.

Polyclonal antibodies which recognize most of the aflatoxin metabolites were produced by Groopman et al 1984. These polyclonals are often used in developing immunoassays like ELISA and RIA.

FIG 4



MONOCLONAL ANTIBODIES

The alternative approach to the production of antibodies is the development of stable hybridoma cell lines that secrete reagent quality monoclonal antibodies. In monoclonal antibodies the short live B-lymphocyes from the spleen are individually immortalized by fusion with immortal myeloma cell line to give a range of clones of B-lymphocytes each which produces its own antibody. This is achieved by isolating single splenic lymphocyte that produces a single antibody that reacts with single epitope, and is of a defined class (IgG) and subclass, from immunized mouse and fusing these with myleoma cell line . Following a series of selection of screening steps, an immortalized clone that constantly produces antibodies of desired affinity, specificity and performance characteristics can be isolated. Monoclonal antibodies for aflatoxin metabolites and aflatoxin lysine adducts were produced by Groopman et al 1996. These monoclones are usually used for immunoaffinity coloums for concentration of aflatoxin adducts.

For aflatoxin exposure assessment polyclonal antibodies are preferred when compared to monoclonals because, the former show certain amount of cross reactivity between various aflatoxin metabolites and thus give more cohesive data about the amount of biologically effective dose of aflatoxin present in the exposed subject. (Wild et al 1990).

2.7.5 Techniques

Using antibodies various researchers developed different immunological techniques like Immuno affinity columns, Radio immunoassay and Enzyme linked Immuno sorbant Assay (ELISA). Initially monoclonal antibodies were used for developing immunoaffinty columns for concentration of aflatoxin adducts in sample and then the sample was analyzed using HPLC. (Kussak et al 1994). Later a sensitive and reliable ELISA was developed by using

polyclonal antibodies by Wild et al 1990 . Radio immuno assay though used by several researchers, utilization of radioactive isotopes and expensiveness restricted its usage.

2.8 E L I S A

The Enzyme Linked Immunosorbent Assay (ELISA), (Enzyme Immunoassay (EIA) or Solid-Phase Immuosorbent Assay (SPIA)) is a sensitive laboratory method used to detect the presence of antigens (Ag) or antibodies (Ab) of interest in a wide variety biological samples. Many variations in the methodology of the ELISA have evolved since its development in the 1960s but the basic concept is still the immunological detection and quantitation of single or multiple Ag or Ab in a patient sample (usually serum).

2.8.1 Types of ELISA

Direct ELISA

Direct ELISA is the most basic of ELISA configurations. It is used to detect an Ag (virus/bacteria/fungus, recombinant peptide/protein, or another Ab) after it has been attached to the solid phase (eg. a membrane or polystyrene microwell or dipstick).

An Ab conjugated with a label (yellow star; eg. HRPO, AP, Pencillinase) is then incubated with the captured antigen. After washing off excess conjugate and incubating with a substrate and chromogen, the presence of an expected colour indicates a specific Ab-Ag interaction. The conjugate could be a commercial prepartion specific for the Ag of interest, or an in-house conjugated monoclonal or polyclonal Ab, or even patient serum.

Indirect ELISA

Initially Ag is adsorped onto a solid phase. The first, or primary Ab is incubated with the Ag, then the excess is washed off. A second or secondary Ab, the conjugate, is then incubated with the samples.

The excess is again removed by washing. For colour to develop, a primary Ab that is specific for the Ag must have been present in the sample (eg. human serum, CSF or saliva or the supernatant from a hybridoma culture). This indiactes a positive reaction.

It is important, during assay optimization, to ensure that the secondary Ab does not bind non-specifically to the Ag preparation or impurities within it, nor to the solid phase.

Antigen Capture ELISA

In this, more specific approach, a capturing Ab is adsorbed onto the solid phase. The capture antibody may be the reagent to be tested (eg. the titre of a patients immune response to a known Ag). However, the Ab may be a standard reagent and the antigen the unknown(as when a patients serum is being investigated for the presence of a microbial infection).

The same stringent optimization is required as for Indirect ELISA. This will ensure that the Ab do not cross-react in the absence of Ag, or non-specifically bind to the solid phase.

It is also important, when detecting the Ag, to use Ab from different animal species to prevent same-species Ab binding (eg. a polyclonal rabbit capture Ab will capture a monoclonal conjugate if it was raised in rabbits. This will produce a positive result in the absence of Ag).

Antibody Capture ELISA

In this approach, a capturing Ab is adsorbed onto the solid phase. The Ab is designed to capture a class of human Ab (eg. IgG, IgA or IgM). Next, the sample is applied, containing the Ab under investigation. After washing, an Ag specific for the Ab is added and finally an anti-Ag conjugate provides the signal.

Competitive or Blocking ELISA

In a competitive ELISA, a patient serum and an Ag-specific conjugate are co-incubated with a captured Ag. The amount of colour developed is inversely proportional to the amount of Ag-specific patient Ig present. Careful standardization is required to interpret the results.
In a blocking ELISA, the patient serum is added first, incubated and the excess washed off. Next an Ag-specific conjugate is added and the results interpreted as above. Titres here may be lower if the conjugate is of a high enough titer to displace patient Ab. In a variation of this format, a conjugated Ag is used the competitor.

The following factors play an important role in all types of ELISA

Coating Of Antigen/Antibody To The Solid Phase

The conditions used for the coating of the solid phase with Ag or Ab can seriously effect the outcome of the assay. Some of the variables to be considered include the (i) temperature used for coating, the (ii) concentration of Ag, the (iii) type of buffer, the (iv) time of incubation of the antigen with the solid phase and the (v) type of solid phase used for the ELISA.

(i) Coating Temperature

It has been reported for some antigens that a period of incubation at 37 ^{0}C followed by incubation overnight at 4 ^{0}C provided the best results. Higher incubation temperatures (up to 56 ^{0} C) can provide more beneficial adsorption of some hydrophobic antigens, in a shorter period of time however deterioration of structure can occur at higher temperatures. Overnight incubation at 4 ^{0}C is common and usually provides an adequate coating for non-

commercial assays. For urgent testing, coating for 2 hours at 37 °C usually provides an adequate result. Ensure your anitigen preparation is free or protected from protease's and yeast in this case. Antigens used for the production of commercial kits must also be prepared in such a way that they will retain their antigenicity following any steps needed to increase the shelf-life of the kits. These steps include dehydration of the plates after coating, and the addition of anti-bacterial/anti-fungal or chemical coupling (an extra step in bonding the Ag to the plate) agents.

(ii) Concentration of the Antigen

The assay is ideally designed if the maximum number of binding sites on the solid phase are taken up by an Ag which is in the correct conformation to optimally bind Ab. However, as the concentration of Ag in the coating buffer increases, so does the rate of desorption of Ag from the matrix, during the assay. The steric conformation of the Ag may also be altered at high Ag concentrations, thus reducing Ab binding and increasing the rate of Ag desorption during the assay. The optimal Ag concentration must therefore be determined through the use of a chequer board titration of diluted Ag Vs known positive and negative reference sera.

(iii) Type of Buffer

The buffer composition is usually based on carbonate buffer (e.g. 10-50mM, pH 9.6) Tris.HCl (pH 8.5) or simply PBS (pH 7.2).

(iii) Conjugates and Related Reagents

Horseradish peroxidase (HRPO or HRP) is the most common enzyme conjugate to an antibody in ELISA. Alkaline phosphatase (AP) is also common, and less often ß-galactosidase, urease and glucose oxidase have been used.

(iv) Reaction Substrates

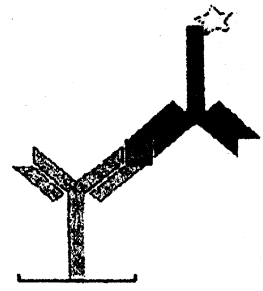
The substrate An ideal substrate should have a defined absorbance peak, be non-carcinogenic, produce a maximal colour in a minimal amount of time and should remain stable during periods of storage - especially useful for commercial kits. Para Nitro Phenyl Phosphate is a widely used substrate for enzyme like Alkaline phosphatase .

Wild et al 1990 presented an excellent overview on the utility of ELISA for aflatoxin exposure assessment. They compared results of ELISA to that of HPLC and confirmed that for population studies hydrolysis ELISA is comparatively better as it gives an opportunity to measure minor serum aflatoxin adducts along with major AFB_1-lysine adduct thus giving holistic value of the effective biological dose. Recently Sashidhar et al 2001 validated indirect ELISA for AFT-Lys measurement. The same procedure was adapted for developing the current assay system.

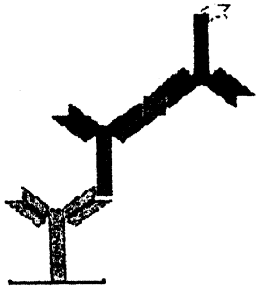
Different types of ELISA

Direct ELISA

Indirect ELISA



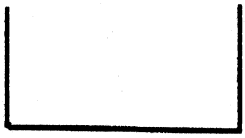
Antigen Capture ELISA



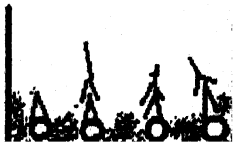
Antibody capture ELISA

Competetive and blocking ELISA

SEQUENCE OF STEPTS IN INDIRECT COMPETETIVE ELISA

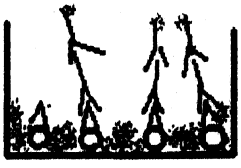


EMPTY WELL



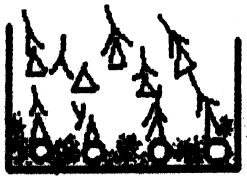
PRIMARY ANTIBODIES ATTACHED TO TOXIN IN PLATE

WELL ADSORBED WITH ANTIGEN-PROTIEN COMPLEX



BLOCKED PLATE

ADDITION OF SUITABLE SUBSTRATE



ADDITION OF FREE TOXIN AND ANTIBODIES FOR COMPETETION

DEVELOPMENT OF COLOUR AND OD MUST BE RECORDED.

KEY:

CHAPTER III
Materials and Methods

3.1 Apparatus

(a.). Pre coated thin layer chromatographic plates – precoated polyester silica gel plates plates (size 20 x 20 cm; particle size 2-25μm sigma chemical co., St.Louis, MO).

(a). Microtiter plates – NUNC Germany .

(b). Spectrophotometer- Beckman DU50, Beckman Co.

(c). Microcentrifuge- CHERMLE Z360k- Forma Scientific Co.

(d). ELISA reader- Titrex MULTISKAN PLUS.(96 well reader).

3.2 Chemicals and Reagents.

All chemical were procured form Sigma Chemical Co.USA

Chemicals:

Aflatoxin B_1 (AFB_1)-
Bovine Serum Albumin
Ovalbumin-
N-α-acetyl –l-lysine,
Goat anti rabbit IgG ,
Para NitroPhenyl Phosphate
MCPBA(m chloro per Benzoic Acid) and
Dichloromethane.(ICN Biomed INC.USA)

3.3 Buffers (composition in Appendix)

Sodium phosphate buffer- sodium phosphate .0.1 M(pH 7.2)
Coating buffer- Carbonate buffer 0.1M pH 9.6
Blocking buffer – 0.5% skim milk in sodium phosphate buffer
Washing buffer – PBS with Tween 20
Substrate buffer- 1 mg/ml – para nitro phenyl phosphate in 10% diethnolamine. (pH 9.8).

3.4 Animals

Rabbits – female (In bred New Zealand white strain. 13 months old. (2-2.5kgs)
Female (New Zealand white strain 2 years old (3-3.5 kgs

All reactions were performed under subdued light to avoid formation of photo adducts. All the reactions must be carried out in glass tube with a glass bead .

Enough Safety precautions were taken to avoid direct contact with toxic substances

3.5 Conjugation of Lysine to Aflatoxin molecule

AFT-Lys adduct was synthesized chemically in two steps. In the first step, aflatoxin molecule was converted to aflatoxin -epoxide and in the next step lysine was attached to the generated epoxide.

Generation of Aflatoxin B_1 -epoxide

0.6 mg of 60% MCPBA was dissolved in 250µl of Dichloromethane. This mixture was washed thoroughly three times with 0.1M PBS . To this, 100 µg AFB_1 dissolved in 250µl of dichloromethane was added and the reaction was allowed to proceed by gentle stirring at 5 ^{0}C for 100 min.

Reaction with Lysine

After stipulated time 1 mg of N-α-acetyl –l-lysine dissolved in 250µl of PBS was added and for another 60 min, the reaction was continued. After one hour, the complete mixture was centrifuged for 5 min at a speed of 10000 rpm. The organic phase (dichloromethane fraction) was separated form the aqueous phase (buffer fraction). The buffer fraction was washed thoroughly with dichloromethane to remove unreacted AFB_1. Both organic fraction and the buffer fraction were analyzed for the presence of AFT-Lys adducts and unreacted AFB_1. The concentration of the adduct was determined using UV spectroscopy. The standard OD value obtained by 1 mg/ ml AFT-Lys adduct as determined by Sabbioni etal 1993, at 343 nm was 7.42 for 1 cm path in a quartz crystal in 0.1 M buffer with pH between 7.2 and 7.4

3.5.1 Characterization by TLC.

Polyester silica gel TLC plates were used for analysis of buffer and organic fraction. The plate was developed in Chloroform –Acetone (9 + 1) solvent system. The florescence of the resulting compound was visualized under long wave UV light (365 nm) in an UV cabinet.

3.5.2 Spectral Analysis of AFT- Lysine for Quantification

Spectral analysis of the adduct was performed using a Beckman DU 50 recording spectrophotometer. The sample was scanned from 200- 500 nm using PBS buffer pH 7.2 as a blank. The absorbance was also taken at 343 nm to quantify the concentration of AFT-Lys Adduct.

3.6 Results and Discussion

3.6.1 TLC

TLC analysis of the buffer fraction of the reaction mixture showed a single fluorescent spot at the base (Rf = Zero) indicating the formation of the Aflatoxin-Lysine adduct. Free unreacted AFB_1 was not detected as compared to the standard. The organic fraction showed different fluorescent spots corresponding to aflatoxin-diols and hydroxyl esters, including unreacted AFB_1.

3.6.2 Spectral Analysis

The UV spectral graph was similar to that of the graph reported by Sabbioni et al 1993. There were two major peaks obtained one was at 275 nm and 340 nm . the OD value obtained at 343 nm was .09. This corresponds to the concentration of 12.4 μg/ml of AFT-Lys adduct .

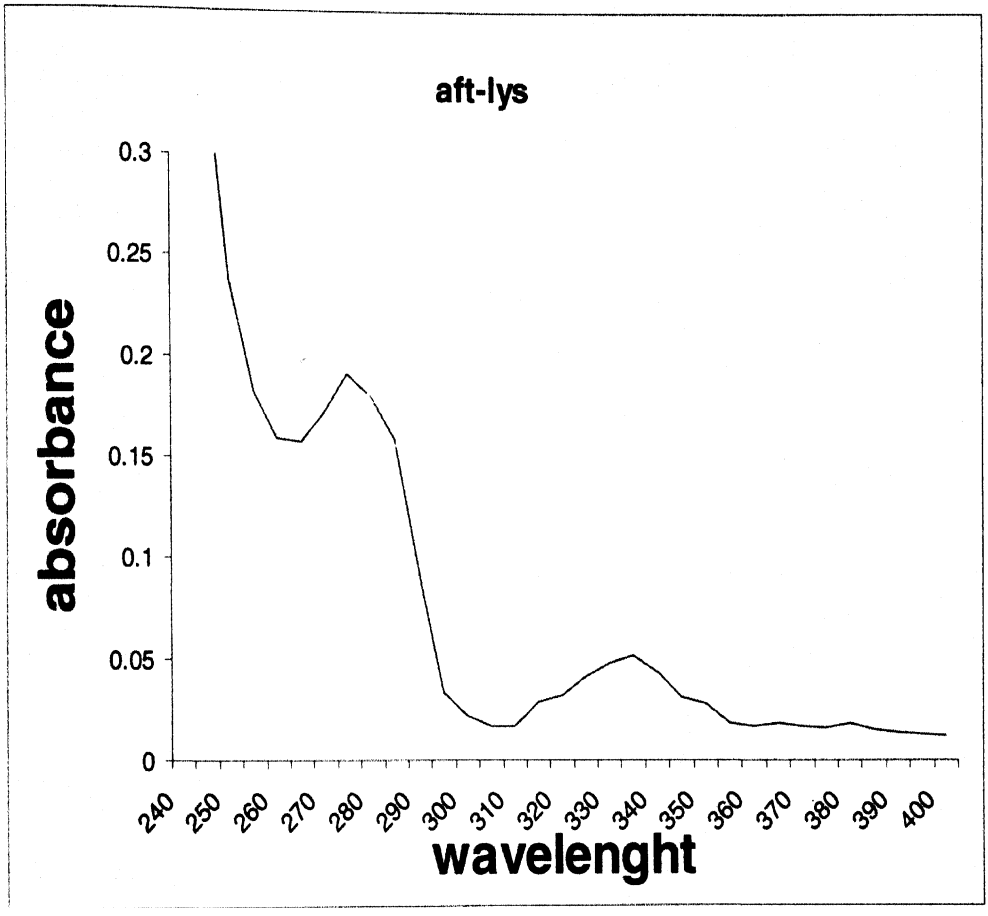


FIG 7 : SPECTRAL GRAPH OF AFLATOXIN LYSINE ADDUCTS

PEAK 1 – 275 nm

PEAK 2- 343 nm

3.7 Synthesis of Aflatoxin – albumin Adducts

AFB1 –BSA adduct was synthesized for raising Polyclonal antibodies against AFB1 epoxide molecule and similarly AFB1 OVA was synthesized as a coating antigen to avoid non specific binding of antibodies to the BSA molecule.

3.7.1 AFB1-BSA IMMUNOGEN

AFB1-BSA was synthesized chemically in two steps. In the first step, aflatoxin molecule was converted to aflatoxin -epoxide and in the next step, BSA was attached to the generated epoxide.

Generation of Aflatoxin B_1 -epoxide

3.6 mg of 60% MCPBA was dissolved in 250µl of Dichloromethane. This mixture was washed thoroughly three times with 0.1M PBS. To this, 588 µg AFB_1 dissolved in 250µl of dichloromethane was added and the reaction was allowed to proceed by gentle stirring at 5^0C for 100 min.

Reaction with BSA

After stipulated time 5 mg of BSA dissolved in 250µl of PBS was added and for another 60 min, the reaction was continued. After one hour, the complete mixture was centrifuged for 5 min at a speed of 10000 rpm. The organic phase (dichloromethane fraction) was separated form the aqueous phase (buffer fraction). The buffer fraction was washed thoroughly with dichloromethane to remove unreacted AFB_1. Both organic fraction and the buffer fraction were analyzed for the presence of AFB1-BSA and unreacted AFB_1. The concentration of the adduct was determined by analyzing the total protein content by both Comasine Blue Assay and UV 280 method.

3.7.2 AFB1-OVA

AFB1-OVA was synthesized chemically in two steps. In the first step, aflatoxin molecule was converted to aflatoxin -epoxide and in the next step, OVA was attached to the generated epoxide.

Generation of Aflatoxin B_1 -epoxide

6 mg of 60% MCPBA was dissolved in 250µl of Dichloromethane. This mixture was washed thoroughly three times with 0.1M PBS. To this, 775µg AFB_1 dissolved in 250µl of dichloromethane was added and the reaction was allowed to proceed by gentle stirring at 5 ^{0}C for 100 min.

Reaction with OVA

After stipulated time 5 mg of OVA dissolved in 250µl of PBS was added and for another 60 min, the reaction was continued. After one hour, the complete mixture was centrifuged for 5 min at a speed of 10000 rpm. The organic phase (dichloromethane fraction) was separated form the aqueous phase (buffer fraction). The buffer fraction was washed thoroughly with dichloromethane to remove unreacted AFB_1. Both organic fraction and the buffer fraction were analyzed for the presence of AFB1-OVA and unreacted AFB_1. The concentration of the adduct was determined by analyzing the total protein content by both Commasine Blue Assay and UV 280 method.

Note : It must be observed that during synthesis of albumin adducts , after centrifugation, three different layers are formed, organic fraction (bottom) and the buffer fraction (top) are separated by a filmy insouble layer. This layer corresponds to precipitated protiens. This must be dicarded.

3.8 Results and Discussion

3.8.1 TLC

TLC analysis of the buffer fraction of the reaction mixture of both AFB_1-BSA and AFB_1-OVA showed a single fluorescent spot at the base (Rf = Zero) indicating the formation of the Aflatoxin-albumin adduct. Free unreacted AFB_1 was not detected in both the cases as compared to the standard. The organic fraction showed different fluorescent spots corresponding to aflatoxin-diols and hydroxyl esters, including unreacted AFB_1.

3.8.2 QUANTIFICATION OF SYNTHESISED ADDUCTS

Both commasine blue reagent assay and UV 280 methods gave similar amounts of protien content . The protein content of aqueous phase of AFB1-BSA and AFB1- OVA showed that the quantity of AFB1-BSA was 6.2 mg / ml and that of AFB1-OVA was 3.4 mg/ml. Low recovery of AFT-OVAlbumin can be attributed to the fact that ovalbumin is less soluble in buffer when compared to the BSA. This can also be observed by the amount of precipitate generated in respective cases.

3.9 Production of Polyclonal Antibodies against AFB1-BSA

Polyclonal antibodies against AFB1-BSA were produced in a single Inbred New Zealand white rabbit (thirteen months old, body mass 2.5-3 kg). A primer dose of 250 µg / ml of AFB1-BSA was given subcutaneously at five different places after through emulsification in complete Freud's adjuvant.

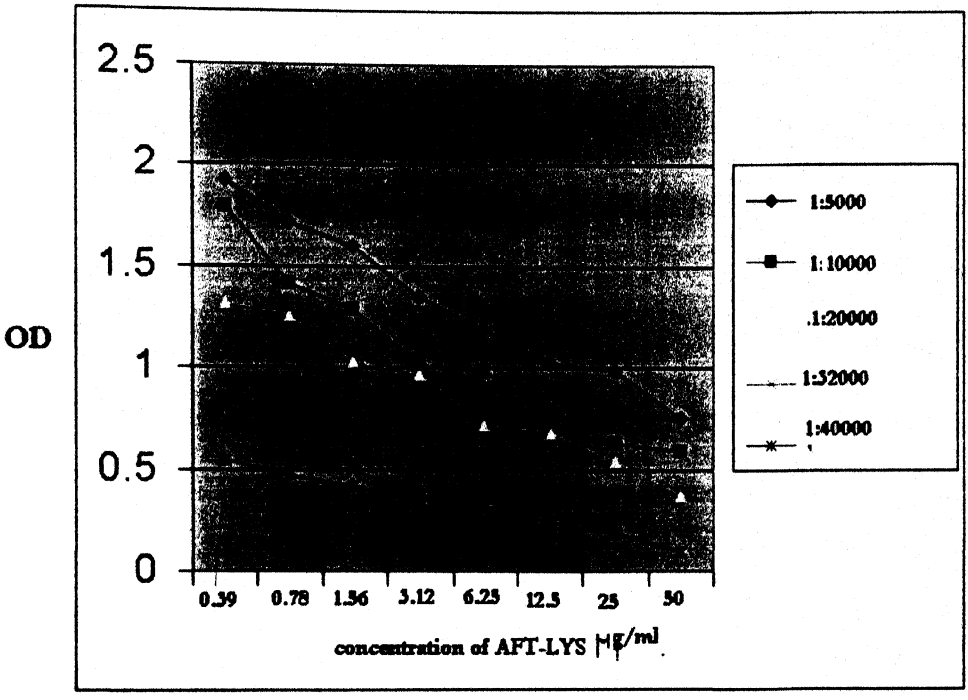
The subsequent doses were placed at an interval of one week where 300µg/ml of AFB1-BSA was administered subcutaneously after through emulsification in incomplete Freud's adjuvant. The antibody titers are being monitored on regular intervals of time and the first booster dose of 300µg/ml was administered after a dip in antibody titer was noticed.

3.10 Monitoring Titers of Antibody.

An indirect ELISA procedure was used to monitor the antibody titers. Microtiter plates were coated with 0.2 μg/ml of AFB1 – OVA (to avoid interference due to antibodies specific to carrier protein BSA) in 0.2 M sodium carbonate buffer, pH 9.6 (150μl/well) and incubated overnight in a refrigerator. Subsequent steps were performed at 37^0 C for one hour. The wells were blocked for nonspecific binding with 165μl/well using blocking buffer (4% dried milk prepared in phosphate buffer saline containing 0.05% Tween-20. Anti serum dilutions in 50μL/well were added to 100μL of AFT-Lysine at concentrations ranging from 50 ng /ml to 90 pg /ml . Goat anti rabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect antibodies attached to AFB1-Lysine. P-nitrophenyl phosphate was used as a substrate at 1 mg/ml and allowed to develop for one hr at room temperature. Asorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titretek Multiskan, Labsystems.). the regression curve was plotted against the optical density at 405 nm and log 10 of the concentration of the toxin. The IC50 was calculated by dividing OD value at 0 toxin level and OD value at 50ng/ml (OD_0/OD_{50})

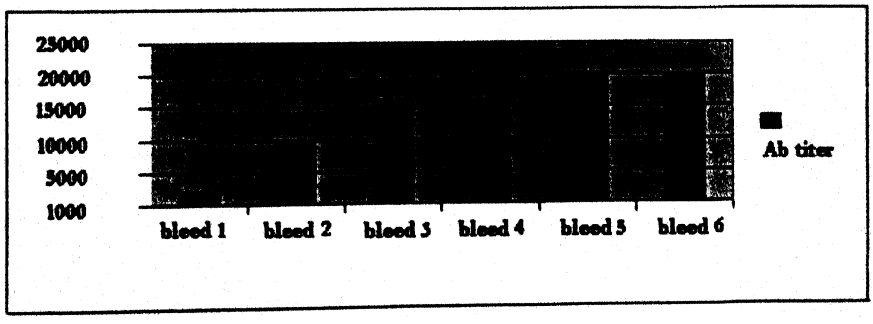
3.11 Results

The the rabbit responded moderately to the given immunogen. The initial titer of the antibody was measured after 5 doses of the immunization schedule . a titer value of 1:2000 gave a regression curve with corelation coeffecient value of 0.993. The titers were monitered on weelky basis. At titer value of 1:16000 was reached on fourth week and was stable during subsequent bleeding . After a booster due the titer value increased to 1:20000 and is stable till date. The subsequent titiers are being monitored. The regression curves showed a corelation coefficient (r^2)0.99 which was reproducible. The inhibitory concentration IC 50 was obtained at 6 ng / mL .



Regression curves for different Ab concentrations

MONITORING OF ANTIBODY TITER



3.12 Estimation of Aflatoxin-Lysine adducts in samples

Bovine blood samples were collected for local diary farm. A total of 41 samples were collected and 38 samples were analysed for the presence of Aft-lysine adducts. The blood samples were collected under the supervision of experienced personnel.

A positve control was obtained form a rabbit which was fed with 100µg/Kg of aflatoxin contaminate peanut diet which was directly gavaged in to the mouth for a week . The blood was taken from the rabbit by vien puncture of the ear pinna . A negative control was bovine albumin comercially available.

Serum Storage

Serum form all the blood samples was separated(annexure) and was stored at –20 ^{0}C till they were further analysed .

3.12.1 Sample preparation

All the boold samples were subjected to the following procedure for separation of aflatoxin lysine adducts.

Separation and Estimation of Albumin

Form 500µl of serum of each sample albumin was extracted and quantified using UV 280 method. The procedure followed for albumin extraction and quantification is mentioned in annexure.

Enzymatic digestion and separation of AFT-Lys adducts

2mg of albumin was taken form each sample and was subjected to digestion with 0.67mg of protienase k overnight.. Then 2 volumes of cold ethanol was added to each samples. Before

adding ethanol 10 mg of BSA was added to enhance the precipitation of undigested albumin and protienase K . the complete mixture was kept at –20 ^{0}C for 2 hours and then was centrifuged at 1000 rpm for 10 min . the supernatant was collected and was left over night at 37^{0}C for the evoparation of the ethanol content.

Now the remaining solution was subjected to ELISA for quantification of the Aft-Lys adducts .

3.13 Quantification of Aft-lysine Adducts

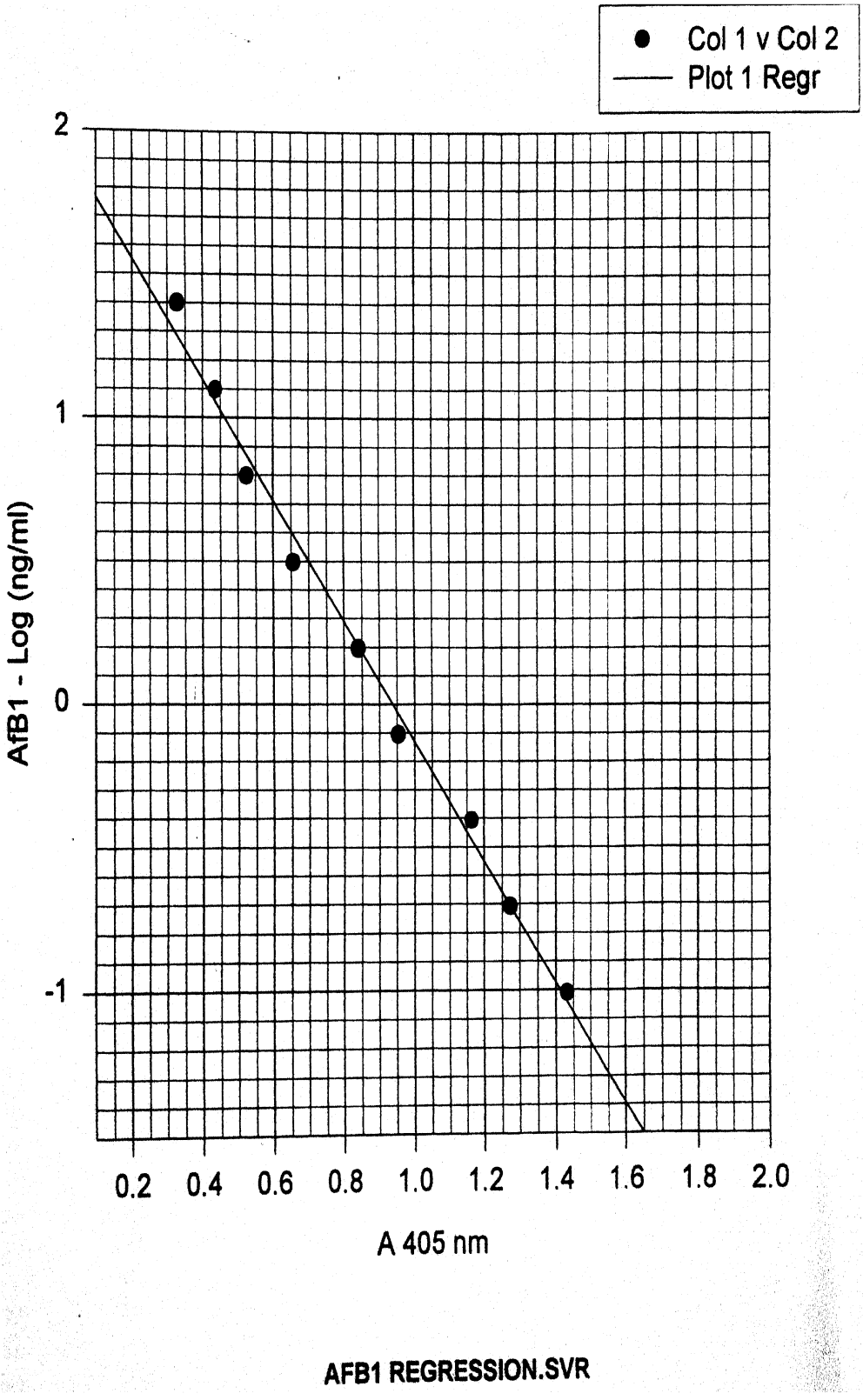
An Indirect cometetive ELISA was performed for quantification. The protocol used was similar to the one used for determination of antibody titers. In brief ELIZA plated were coated by incubating AFT-OVA conjugate in 0.2 M sodium carbonate buffer pH 9.6 (150µl/ well) at a concetration of 200ng/ml in a refrigrator overnight. Subsequent steps were perormed at 370c for 1 hr. the Plated were bolcked using blocking buffer. Simultaneously antibody dilutions prepared in phosphate buffered saline and 0.2% BSA were incubated at 37^{0}C to cross absorb antibodies against BSA . This step was performed to avoid any cross reaction as bovine samles are being estimated. Toxin standards ranging form 25ng/ml to 50pg/ml were prepared . The amount of antibody bound to the toxin was detected by addition of anti rabbit IgG immunoglobulin conjugated to alkaline phosphatase enzyme . the substrate for ALP , p-nitrophenyl phospahte was used at 1 mg/ml. Colour was allowed to develop at room temperature and then absorbance of the hydrolysed product was measured at 405nm in an ELISA plate reader. Standard curves were obtained by plotting Log_{10} values of toxin standards againt optical density at 405 nm and was expressed in ng/mg .

3.14 Results and Discussion

The albumin levels of each sample and the amount of aft-lysine adduct are shown in table

All the blood samples analysed were positive for the presence of aft-lysine adducts. The mean adduct level was 6.25ng/mg of albumin . The least value obtained was 0.02ng/mg of albumin where as the highest value was 11.04ng/mg of albumin. The amount of Aflatoxin Albumin adducts keep on accumulating inside the exposed individual and thus give a picture of long term exposure. This indicates that all the animals are being exposed to aflatoxin in their diet on a regualr basis. This will have a negative effect on the health of the exposed animals . Moreover consumption of milk from such farms may effect the health of humans, if as chances of AFM1 contamination in milk is quiet high .

As this is a newly developed detection methodology, further conformation / or support of another detection stystem such as HPLC must be used to analyse the blood samples so that a correlation can be extablished between the two techniques and any false positive reactions can be detected , which is possible with immunological detection methods.



AFB1 REGRESSION.SVR

Table 3 : **AFT-Lys in blood samples**

serial No.	sample r	albumin content	sample(mg)	E=(2*700/C)	aft-lys(ng/mg)
1	687	14.46	2	96.5	5.75
2	125	13.58	2	103	6.36
3	825	14.05	2	99.6	9.155
4	844	13.1	2	106	6.9
5	687	14.4	2	97.2	6.68
6	612	13.35	2	104.2	0.075
7	875	15.04	2	93	7.435
8	710	13.35	2	104.5	7.33
9	301	10.8	2	130	0.09
10	740	22	2	63.6	10.85
11	874	10.1	2	139	4.27
12	243	10.5	2	133.3	6.9
13	828	14.9	2	94	9.5
14	516	10.2	2	138	6.92
15	309	13.56	2	103	6.65
16	546	13.3	2	105.26	7.24
17	461	12.5	2	112	6.39
18	187	15.3	2	91.5	8.77
19	346	11.35	2	123.5	7.95
20	888	14.5	2	96.5	0.13
21	466	11.9	2	117.6	7.27
22	26	12.2	2	114.5	8.03
23	895	10.2	2	137	8.25
24	469	10.9	2	128.5	7.9
25	624	15.5	2	90.32	0.535
26	300	21.3	2	65.7	0.02
27	174	15.9	2	88	11.04
28	117	12.1	2	115	7.1
29	31	16.5	2	85	0.58
30	7096	12.8	2	109	7.275
31	71	9.8	2	142	6.69
32	308	10.4	2	134.5	5.84
33	365	22.4	2	62.5	0.02
34	682	24.5	2	57	0.025
35	621	12.5	2	112	0.075
36	785	15.7	2	89	0.28
37	298	10.8	2	130	9.68
38	388	25.6	2	55	8.96
39	rabbit	8.85	2	158	28.8

Chapter IV

Scope of Future Work

Aflatoxin exposure is being considered as a big problem in almost all-developing and developed countries. A number of population studies are being carried out in middle east countries like China, Philippines, Thailand and in places like Benin, Zambia in West Africa..In all the above countries, a strong relation has been established between aflatoxin exposure, prevalence of Hepatitis B viral infection and primary hepatocellular carcinoma. However, unfortunately in Indian subcontinent where aflatoxin contamination is quite common due to favorable environmental conditions and poor storage facilities, no such population studies are being carried out. Such studies must be done in Indian subcontinent especially in those areas where aflatoxin contamination is very high. This will help in risk assessment of liver cancer development among populations and thus reliable measures can be taken to mitigate the ill effects. All the areas where aflatoxin exposure is being taking place can be mapped and suitable action plans can be devloped for management of aflatoxin right form the field level.

2. ELISA is a very sensitive and highly reliable system. Till date this system is mostly restricted for detection of disease in humans and biological samples. Nevertheless, similar ELISA based systems can be developed for analysis of persistent organic pollutants and dioxins and both biological and environmental samples. Such systems not only gives rapid but also accurate results. Moreover they are cost effective and technical expertise can be achieved quite easily. Dietary exposures to common pesticides are another severe problem which is leading to many health effects. Though stringent regulatory measures are being employed, indiscriminate use of banned pesticides is still being practiced. Such pesticides can be easily detected to minute quantities in both soils and foodstuffs using ELISA based systems.

Chapter V

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And

APPENDIX

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Appendix

BUFFERS

Carbonate Buffer (Coating Buffer)

Na_2CO_3	1.59 grams
$NaHCO_3$	2.93 grams
D . H_20	2 liters

PH adjusted to 9.6

Sodium Phosphate Buffer

NaH_2PO_4 (1 M)	31.6 mL
Na_2HPO_4 (1 M)	68.4 mL

Ph 7.2

Phosphate Buffer Saline Tween or Wash Buffer

NaH_2PO_4	2.8 gms
KH_2PO_4	0.4 gms
KCl	0.4 gms
Na Cl	16 gms
Tween 20	1 ml
Distilled water	2 liters

Blocking Buffer

0.5% Skim Milk Powder in Phosphate buffer.

Separation of Albumin form Blood

500µl sera preheated in H2O bath (56°C) for 45 min. to inactivate any HIV virus present. Cool on ice for 15 min
Add 1.5 volumes of cold saturated ammonium sulfate (usually 60%) slowly.
Vortex the Mixture and centrifuge at 9000g, 4°C for 15 min. to remove immunoglobulins.
Supernatant contains the albumin—removed and 100µl of 1M acetic acid was added to adjust the pH to 5.
Centrifuge at 9000g, 4C, 15 min. and collect the precipitated albumin.

Coomassie dye binding assay or Bradford protein concentration assay

Reagents:

Dye stock--- 100 mg of Coomassie Blue G is dissolved in 50 ml of methanol. The solution is added to 100 ml of 85% H3PO4, and diluted to 200 ml with water. The solution should be dark red, & have a pH of –0.01. The final reagent concentrations are 0.5 mg/ml Coomassie blue G, 25% methanol, and 42.5% H3PO4. The solution is stable indefinitely in a dark bottle at 4C.

Assay reagent---the assay reagent is prepared by diluting 1 volume of dye stock with 4 volumes of distilled H2O. The solution should appear brown, and have a pH of 1.1. It is stable for weeks in a dark bottle at 4C.

Protein standards--- Protein standards should be prepared in the same buffer as the samples to be assayed. A convenient standard curve can be made using BSA with concentrations of 0, 250, 500, 1000, 1500, 2000µg/ml for the standard assay and 0, 10 , 20, 30 , 40, 50 µg/ml or 0, 5, 10, 15, 20, 25µg/ml.

Dilution of the protein sample may be required for the resulting absorbance to fall within the linear range of the assay.

Allow each sample to incubate at room temperature for 10-30 min..

Measure the absorbance of each sample at 595 nm using a uv-visible spectrophotometer.

Preparation of test samples for the Bradford Protein assay

Test Sample	Sample volume (µl)	Vol. Water (µl)	Vol. Bradford reagent (µl)
Reference cell	0	800	200
Blank	0	800	200
BSA standard- 5µl/ml	10	790	200
BSA standard- 10µl/ml	20	780	200
BSA standard- 15µl/ml	30	770	200
BSA standard- 20µl/ml	40	760	200
BSA standard- 25µl/ml	50	750	200
Protein sample	50	750	200